POROS® 50 MC
Perfusion Chromatography®
Bulk Media for
Metal Chelate Chromatography

 Applied Biosystems

Operating Instructions

NOTE: These instructions cover the specific operational characteristics of POROS® 50 bulk media. The same surface chemistries are available as bulk media with 20-µm particle size (POROS 20 bulk media), as well as in prepacked columns. Contact your Applied Biosystems representative for more details.

Your New POROS 50 Media Is Unique

Read this section before doing anything!

Applied Biosystems POROS 50 bulk media are based on 50-µm flow-through particles. POROS 50 media allow you to use Perfusion Chromatography® technology under low operating pressures. Chromatographic separations of biomolecules can be performed considerably faster than conventional liquid chromatography separations, while maintaining high resolution and high binding capacity.

Although columns packed with POROS 50 media can be operated with standard low-pressure and high-pressure systems, they are substantially different from any columns you have used before. You may have to change the way you run, and, to a large extent, the way you think about chromatography. In particular, the higher flow rates made possible by Perfusion Chromatography media allow you to perform experiments you might once have considered a luxury, given the constraints of classical chromatography's longer run times.

With Perfusion Chromatography media, you now can perform true methods development by systematically investigating a wide range of chromatographic variables in a short time frame. In addition, the higher flow rates translate into significant gains in throughput and processing capacity when scaling up to production.

Please read the operating instructions carefully to ensure that you take maximum advantage of the benefits that Perfusion Chromatography technology provides.

Increase the Flow Rate

The largest single difference between POROS media and conventional media is the flow rates at which experiments are run.

While the media can be used at flow rates typical of conventional chromatography, you can realize the full benefits of Perfusion Chromatography with POROS 50 MC media only by increasing the flow rate so that the linear velocity is in the range of 800 to 1,000 centimeters per hour (cm/h).

The recommended starting flow rate range for POROS 50 MC media is 500 cm/h. Depending on the sample material and the experimental conditions, you can then increase the flow to maximize processing capacity [(grams of product per liter of column volume)/hour].

Linear velocity (cm/h) is calculated by dividing volumetric flow rate (cm^3/min) by the column cross-sectional area (cm^2) and multiplying by 60 min/h.

The dramatically higher flow rates of POROS columns and media introduce new considerations into the design and implementation of experiments. This is particularly true when you adapt a method developed on diffusion-limited media.

Be sure to read Section 9, Guidelines for Using Perfusion Chromatography, for a full discussion of these considerations. Another excellent reference is *The Busy Researcher's Guide to Biomolecule Chromatography*, available from your Applied Biosystems Technical Representative.

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1 Product Description

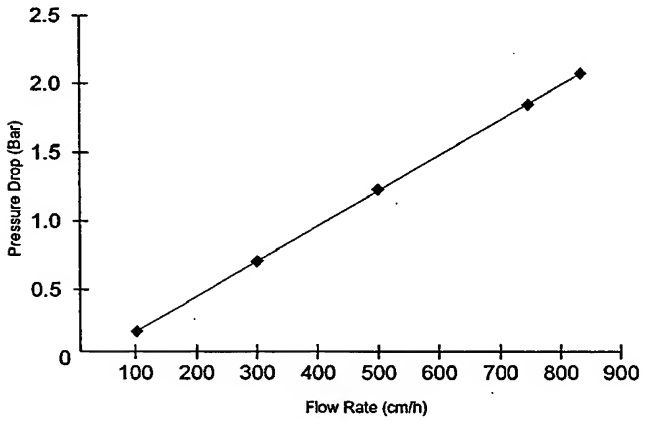
POROS MC media is a polymeric packing designed for immobilized metal affinity chromatography of peptides, proteins, and other biomolecules in the Perfusion Chromatography mode. The packing consists of cross-linked poly(styrene-divinylbenzene) flow-through particles with a patented bimodal pore size distribution for very rapid mass transport.

Particles are surface-coated with a cross-linked polyhydroxylated polymer functionalized with imidodiacetate groups. This functionality allows binding through bidentate ligation of a wide range of transition metals. Binding to proteins is by the formation of coordination complexes between the remaining metal coordination sites and certain surface amino acids, particularly histidine, cysteine, and tryptophan.

Table 1 Product Characteristics

Support Matrix	Cross-linked poly(styrene-divinylbenzene)
Surface Functionality	Imidodiacetate ($-CH_2N(CH_2CO_2^-)_2$)
Dynamic Binding Capacity @ 360 cm/h	Myoglobin, Cu^{2+} form 15 mg/mL
Metal Capacity	65 µmol/mL (Cu^{2+})
Particle Size	50 µm
Recommended maximum flow rate	1,000 cm/h
Maximum pressure drop	103 bar (1,500 psi, 10 MPa)
Permeability	<3 bar at 1,000 cm/h (10 cm bed height)

POROS 50 MC media is mechanically stable up to backpressures of 103 bar (1,500 psi). Therefore, bed compression at high flow rates is not a concern. The pressure-flow properties of POROS 50 MC media (Figure 1) also allow the media to be packed and run using conventional low-pressure columns and systems.



Column geometry: 25 cmD/10 cmL
Packing pressure: 3 bar

Figure 1 Pressure-Flow Properties of POROS 50 Media

Table 2 Chemical and Thermal Resistance

pH Range	1 to 14
Ionic Strength Range	0 to 5 M, all common salts
Buffer Additives	All common agents, including 8 M urea, 6 M guanidine hydrochloride, ethylene glycol, and detergents. Agents that may degrade the ligand are not recommended.
Solvents	Water, 0 to 100% alcohol, acetonitrile, other common organic solvents *Note: Do not expose to strong oxidizers (such as hypochlorite), oxidizing acids (such as nitric), or strong reducing agents (such as sulfite).*
Operating Temperature	5 to 40 °C

2 Packing the Column

This section discusses:

- Packing solvents
- Preparing the slurry
- Packing procedures

POROS 50 media are mechanically rigid and, therefore, can be packed effectively both in low-pressure glass columns and in high-pressure stainless steel columns. The frit size must be less than 15 µm.

Keep the media container closed when it is not in use.

2.1 Packing Solvents

Use these solutions:

- Slurry buffer: 0.5 M NaCl
- Packing buffer: 0.1 M NaCl

2.2 Preparing the Slurry

⚠ WARNING CHEMICAL HAZARD. POROS 50 MC Slurry containing ethanol *is a flammable liquid and vapor. It may cause eye, skin, and respiratory tract irritation. Prolonged or repeated contact may dry the skin. It contains material that may cause central nervous system depression and liver damage. Keep away from heat, sparks, and flame. Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

POROS 50 MC media, which is shipped as a slurry containing 20% ethanol as a bacteriostat, yields the final packed-column volume indicated on the label. Calculate the total volume of slurry needed to pack your column by keeping in mind that the ratio of slurry volume to packed-column volume is 1.8:1.0.

For example, you need 18 mL of slurry to pack a 10-mL column volume. The packed-column volume specified on the label is based on a packing pressure of 3 bar.

To prepare the slurry for packing:

1. Allow the media to settle for 3 hours.
2. Pour off the supernatant.
3. Resuspend the media in 0.5 M NaCl.

   The volume of 0.5 M NaCl to add depends on the column equipment you use. In general, the final slurry volume should be 2 to 3 times the final packed-column volume.

   Note: Do not use a magnetic stirrer. It may abrade the particles and cause fines to form.

2.3 Packing Procedures

To ensure best results when you pack the column:

- Use a reservoir or adjustable column large enough to contain the entire slurry, so that the bed can be packed all at once.
- Use flow-packing or pressure-packing techniques (see below).

Flow Packing

To flow-pack the column:

1. Gently stir the slurry just before adding it to the column.

   NOTE: POROS 50 beads have a skeletal density similar to the density of water, so rapid settling is not a problem.

2. Pour the slurry into the column gradually to minimize the trapping of air bubbles.
3. Tap the column gently to remove air bubbles.
4. Top off the column with the slurry buffer.
5. Prime the packing pump and top adjuster with packing buffer. Remove all air bubbles.
6. Connect the top adjuster to the column.
7. Start the flow slowly, until a clear space forms between the column top adjuster and the slurry.
8. Increase the flow rate to the maximum flow rate and pressure obtainable with the equipment used.

   NOTE: The final packing flow rate should be at least 20% greater than the maximum anticipated operating flow rate.

9. After the bed is formed and the final flow rate is reached, bring the column top adjuster into contact with the top of the bed.
10. Restart the flow for three column volumes to stabilize the bed.

    NOTE: POROS 50 media does not shrink or swell, so an open "head space" is not recommended.

11. Pump the column for five to ten column volumes.

    Use "equilibration run" conditions (no sample applied), including the minimum and maximum ionic strengths used in actual operation.

    The column is ready for operation.

NOTE: You may observe some fine material in the eluent as packing begins. This will clear after two to three column volumes of packing buffer pass through the column.

Pressure Packing

To pressure-pack the column:

1. Gently stir the slurry just before adding it to the column.

   NOTE: POROS 50 beads have a skeletal density similar to the density of water, so rapid settling is not a problem.

2. Pour the slurry into the column gradually to minimize the trapping of air bubbles.
3. Tap the column gently to remove air bubbles.
4. Top off the column with the slurry buffer.
5. Prime the top adjuster with packing buffer.
6. Connect the top adjuster.
7. Expel trapped air from the column by applying 5 psi to the system.
8. Select the packing pressure on the pressure gauge.
9. Pack the column.
10. Apply flow for three column volumes to stabilize the column bed.

    NOTE: The flow rate should generate no more than 80% of the packing pressure.

11. Pump the column for 5 to 10 column volumes.

    Use "equilibration run" conditions (no sample applied), including the minimum and maximum ionic strengths used in actual operation.

    The column is ready for operation.

NOTE: You may observe some fine material in the eluent as packing begins. This will clear as packing proceeds, after two to three column volumes of packing buffer pass through the column.

3 Selecting and Loading the Metal Ion

⚠ CAUTION CHEMICAL HAZARD. *Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

The metal ion loaded on the imidodiacetate chelating groups on POROS MC media has a critical effect on both the binding strength and selectivity for different proteins. Consider the following information as you choose a metal ion:

- The most commonly used ions, in order of greatest to lowest binding strength, are:

  Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} (the last two ions are of about equal binding strength)

- You can also use other metal ions, including Fe^{3+}, Al^{3+}, and Ga^{3+}. Fe^{3+} is unusual in that, unlike other ions, elution can often be effected by increasing pH or increasing concentrations of salt.
- Use Cu^{2+} for initial studies when nothing is known about the characteristics of the protein of interest. Cu^{2+} tightly binds to any protein that can be separated using metal chelate chromatography.

  Then substitute other metal ions to increase selectivity for the protein of interest or for key contaminants, or to reduce binding strength for improved recovery.

3.1 Saturating the Imidodiacetate Sites

Make sure that the imidodiacetate sites on the column are fully saturated with the proper metal. To do this:

1. Before loading the metal, perform a stripping wash with 10 to 20 column volumes of either of the following solutions:
   - 50 mM EDTA, 1 M NaCl
   - 150 mM HCl, 150 mM NaCl
2. Wash with 5 to 10 column volumes of water.
3. Depending on pH levels, do the following:
   - In weakly acidic solutions (pH 5 to 6), load metal ions as sulfate or chloride salts to avoid precipitation of metal hydroxide complexes.
   - In more acidic conditions (pH 2 to 4), load Fe^{3+} because of solubility difficulties at higher pH.

     The concentration of the metal salt is not critical (0.05 to 0.25 M concentrations are generally used).
4. Load at least 0.25 mM metal/mL column volume (50 mL of 0.1 M for a 1.7-mL column).

   With Cu and Ni, you can observe metal breakthrough visually or with a UV detector.
5. Wash with 5 to 10 column volumes of water to remove excess metal.
6. Wash the column with 5 to 10 column volumes of 0.1 to 0.5 M NaCl to remove metals that may be bound ionically.
7. Wash with 5 to 10 column volumes of starting buffer.

NOTE: Wash thoroughly with salt solution between metal loading steps. Any condition with free metal ions in solution can lead to precipitation and column plugging. If plugging occurs, wash the column at a low flow rate with dilute acid to redissolve the metal.

3.2 Stripping and Reloading the Column

Whether or not you should strip and reload the column with metal between runs depends on the metal used and the elution protocol:

- Reloading is essential with weakly complexing ions such as Zn^{2+} or Co^{2+}.
- Cu^{2+}-loaded columns can sometimes be used for many runs without reloading of metal, unless stripping eluents such as EDTA are used.

In all cases, stripping and reloading after every run gives maximum reproducibility.

4 Selecting a Buffer and an Elution Method

Because metal chelate chromatography is not as well developed as more traditional chromatography modes such as ion-exchange or reversed-phase, it is virtually impossible to predict retention behavior. As a result, conditions for binding and elution must often be developed by trial and error.

However, very short run times on POROS MC columns make this development process much faster than with conventional media.

4.1 Starting Buffers

⚠ WARNING CHEMICAL HAZARD. Guanidine hydrochloride *may be harmful if swallowed or absorbed through the skin. Exposure may cause eye, skin, and respiratory tract irritation and adverse effects on the central nervous system and bone marrow. Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

⚠ CAUTION CHEMICAL HAZARD. Urea *may cause eye, skin and respiratory tract irritation. Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

Regardless of the starting buffer and elution method you choose, it is always important to:

1. Use buffers of the highest purity practical.
2. Degas and filter (0.22 or 0.45 µm) all buffers before use.
3. Maintain relatively high ionic strength (0.1 to 1.0 M) throughout.

Follow these guidelines when you select a starting/wash buffer:

- Starting buffers can be in the range of pH 4 to 8.5. Generally, pH 7 to 8 gives the best results.
- Acetate and phosphate buffers often result in strong binding.
- Buffers containing primary amines (such as Tris) often weaken binding and can strip metals, but may still be used in some cases.
- NaCl at 0.1 to 1.0 M is recommended to suppress secondary ionic interactions and protein/protein interactions.
- If the column is run using eluent saturated with elution agent (see Section 4.2, "Elution Method"), the starting buffer should also contain 0.5 mM of the elution agent.
- Chaotropic agents such as guanidine hydrochloride or urea can be used if needed.

4.2 Elution Method

⚠ WARNING **CHEMICAL HAZARD. Imidazole** *is harmful if swallowed. Exposure may cause eye, skin, and respiratory tract irritation. Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

⚠ WARNING **CHEMICAL HAZARD. 2-Methyl pyridine** *is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation. It is harmful if inhaled, absorbed through the skin, or swallowed, and may cause central nervous system depression and damage to the liver and kidneys. Keep away from heat, sparks, and flame. Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

Several methods of elution are possible:

- Reduce the pH to between 3.0 and 6.0.

  Acetate and phosphate buffers are widely used because they can be used to form continuous pH gradients in this range.

  However, reducing the pH frequently does not elute tightly binding proteins and often gives poor reproducibility and resolution. In addition, the pH may pass through the isoelectric point of some sample proteins, causing precipitation.

- Use increasing gradients of solutes that compete with the protein for metal binding sites.

  Increasing the gradient gives better results than reducing the pH. In most cases, imidazole works well in relatively low concentrations (usually 1 to 50 mM), although concentrations as high as 250 mM may be needed.

  In cases where binding is too weak for imidazole to be useful, use 2-methyl pyridine. Also, you can use ammonium salts or glycine in relatively high concentrations (0.1 to 1.0 M), but usually they give poor resolution.

- Use a chelating agent such as EDTA (50 to 500 mM) to remove both the metal ion and the protein.

  A chelating agent always elutes specifically bound protein, and is recommended for regeneration of the column. However, it does not work in gradient mode and cannot be used to separate bound proteins from each other.

4.3 Improving Selectivity and Recovery

For many applications, you can improve selectivity and recovery by saturating the column with the displacing elution agent (such as imidazole) before loading the sample. This replaces water on most of the metal coordination sites with the elution agent and results in a favorable reduction in the binding strength. To do this:

1. After the metal is loaded and free metal washed off, wash the column with 5 to 10 column volumes of the highest concentration of the agent used for elution (or until a breakthrough is observed on the UV detector).
2. Wash with 5 to 10 column volumes of starting/wash buffer. The starting/wash buffer should contain a low concentration (0.5 mM) of the elution agent to keep the column saturated.

5 Preparing and Loading the Sample

To ensure efficient binding and prevent column plugging:

- Dissolve or exchange samples into the starting buffer. It is important to expose the sample to the salt concentration in the starting buffer to remove any components that may precipitate.
- Centrifuge or filter samples (0.22 or 0.45 µm) before injection.
- Delipidate samples, if possible. Lipids can cause irreversible fouling.

***NOTE:** With some crude samples, setting the flow rate between 200 to 350 cm/h during the loading step can increase binding efficiency.*

5.1 Determining the Sample Load

The dynamic binding capacity of POROS MC media is between 5 and 20 mg/mL for most peptides and proteins.

In general, high-resolution separations are achieved at 20% or less of the total binding capacity.

However, the maximum loading at which a given resolution can be obtained (the loadability) depends on a number of factors, including sample solubility, column selectivity, and so on.

The long run times associated with conventional chromatography usually prohibit a systematic determination of loadability. The short run times associated with Perfusion Chromatography media make it easy to perform this determination.

To perform a loading study:

1. Determine the most effective elution conditions (eluent, gradient, and flow) at low loading.
2. Gradually increase the sample load (either through increasing injection volume or sample concentration or both) until the required resolution no longer occurs.

***NOTE:** As the loading is increased, the peaks may elute earlier on the gradient, possibly necessitating reoptimization of the gradient.*

Once again, the short run times made possible by Perfusion Chromatography media make this quick and easy.

5.2 Concentrating Dilute Samples

The binding of protein to POROS MC columns and the resulting elution peak depend on the total mass, not the concentration, in the sample. This is because of the very high binding constants inherent in the protein-ligand interaction. Therefore, the column can concentrate very dilute samples such as cell culture supernatants.

Because of the high flow rates possible with Perfusion Chromatography, concentrating a dilute sample does not require much time. Sample sizes can be as large as 20 to 30 column volumes or more and still give good results in 2 to 5 minutes or less.

6 Regenerating and Cleaning Up the Media

In some applications, sample molecules may not fully elute or may precipitate on the column. Regenerate the column if any of the following symptoms occur:

- Increased bandspreading
- Loss of binding capacity
- Loss of recovery
- Increased pressure drop
- "Ghost" peaks occurring during blank gradient runs

NOTE: In the cleanup method, reverse the flow direction to help flush out particulates and to prevent contamination of the lower part of the bed. Also, slow the flow rate to expose the column to the regeneration solution for several minutes at each step of the cleaning protocol.

6.1 Regenerating the Column

⚠ DANGER **CHEMICAL HAZARD. Sodium hydroxide** *causes severe eye, skin, and respiratory tract burns. Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

⚠ DANGER **CHEMICAL HAZARD. Acetic acid (glacial)** *is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract burns. It may be harmful if inhaled, absorbed through the skin, or swallowed. Keep away from heat, sparks, and flame. Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

⚠ DANGER **CHEMICAL HAZARD. Hydrochloric acid** *causes severe eye, skin, and respiratory tract burns. Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

⚠ DANGER **CHEMICAL HAZARD. Trifluoroacetic acid (TFA)** *causes eye, skin, and respiratory tract burns. It is harmful if inhaled. Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

***IMPORTANT:** In any regeneration step, you need to first strip the column of metal ions. Otherwise, they may precipitate irreversibly and destroy the column.*

To regenerate the column:

1. Strip the column by washing with 1 to 5 column volumes of either of the following solutions:
   - 0.1 M EDTA, 1 M NaCl
   - 150 mM HCl, 150 mM NaCl
2. If stripping the column with either of the above solutions does not restore column performance, complete the following steps in order:
   - Wash with 1 to 5 column volumes of 1 M sodium hydroxide (preferably including 1 to 2 M salt).
   - Wash with 3 to 5 column volumes of water to remove the base.
   - Wash with 1 to 5 column volumes of 1.0 M acetic acid, 1.0 M hydrochloric acid, or 1% trifluoroacetic acid (TFA).
   - Wash with water to remove the acid.
   - Reequilibrate the column with starting/wash buffer.

6.2 Removing Lipids and Lipoproteins

⚠ WARNING **CHEMICAL HAZARD.** *Methanol is a flammable liquid and vapor. Exposure causes eye and skin irritation, and may cause central nervous system depression and nerve damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

⚠ WARNING **CHEMICAL HAZARD.** *Acetonitrile (ACN) is a flammable liquid and vapor. Exposure may cause eye and respiratory tract irritation and blood system damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

⚠ DANGER **CHEMICAL HAZARD. Guanidine thiocyanate** *causes eye burns and can cause skin and respiratory tract irritation. It is harmful if absorbed through the skin or swallowed. Contact with acids and bleach liberates toxic gases. DO NOT ADD acids or bleach to any liquid wastes containing guanidine thiocyanate. Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

To remove fouling lipids or lipoproteins, use any of the following approaches:

- Use a mixture of 50% methanol or acetonitrile with acid or base.
- Use a mixture of 50% methanol and 50% 3 M guanidine thiocyanate.

  NOTE: Take care when using thiocyanate with metal systems. Thiocyanate forms complexes with iron that strongly absorb UV light.
- Store the column overnight in 1 mg/mL pepsin, DNAse, or other enzymes.

6.3 Multiple Injections

You can make multiple injections of regeneration solutions instead of pumping them directly through the column. This method is recommended for very aggressive or very viscous solvents.

To clean by injections:

- Make the injection volume as large as possible.
- Use a low flow rate that exposes the column to the regeneration solution for several minutes.

NOTE: Increased backpressure is sometimes caused by a plugged inlet frit. If backflushing the column does not solve the problem, replace the inlet frit.

7 Storing the Media

To store a prepacked column:

- Carefully seal the ends of the column to prevent drying. Drying can result in decreased chromatographic efficiency.
- Store the column between 4 and 8 °C.

Short-Term Storage

Store the column in any appropriate mobile phase.

Long-Term Storage

⚠ WARNING CHEMICAL HAZARD. *Ethanol is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause central nervous system depression and liver damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

⚠ WARNING CHEMICAL HAZARD. *Methanol is a flammable liquid and vapor. Exposure causes eye and skin irritation, and may cause central nervous system depression and nerve damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

Store the column or bulk media in 0.1 M Na_2SO_4 with any one of these solutions:

- 0.02% sodium azide
- 20% ethanol
- 20% methanol

8 Scaling Up

POROS 50 MC High Capacity media is ideally suited for methods development and scale-up. Follow the guidelines below during development to ensure easy scale-up to preparative and production volumes:

- Program your chromatographic system in column volumes. If this is not possible with your system, make sure that gradient volumes and linear velocities do not change out of proportion to column volume.
- Keep sample loading proportionally the same as column size increases.
- Keep column bed height the same. If this is not practical as you increase column size, make sure that you do not reduce sample residence time as scale increases.
- As column volume increases, if the column maximum pressure limit is reduced, thereby forcing changes in packing pressures or flows, test the effectiveness of packing by measuring HETP and asymmetry using a suitable probe molecule. Protocols are available from your Applied Biosystems Technical Representative

In Applied Biosystems development laboratories, methods are developed at the 10- to 20-mL column-volume scale. Then, method robustness is confirmed with 1-L columns (approximate), followed by scale-up to the required column volume for the application (in many cases hundreds of liters).

NOTE: Column hardware specifications continue to evolve and improve. Contact your Applied Biosystems Technical Representative for the most recent recommendations of column types for your application.

9 Guidelines for Using Perfusion Chromatography

There are a few simple but important guidelines to keep in mind when you make the transition to Perfusion Chromatography. They can be grouped into two general categories:

The chromatography system:

- Account for system pressure
- Check the gradient system
- Adjust the data collection system
- Maintain your column and system

Experimental design:

- Think in terms of column volumes
- Adjust the sample load
- Measure recovery properly

9.1 Account for System Pressure

The high flow rates used with Perfusion Chromatography cause a higher-than-usual system pressure (resulting from the chromatographic hardware itself). In some cases, this system pressure can be equal to or even greater than the column pressure.

Therefore, when you use your POROS column, you cannot simply set the upper pressure limit of the system at the pressure rating of the column. Instead, you need to:

1. Determine the system pressure by:
   - Connecting a union in place of the column
   - Pumping the solvent with the highest salt concentration to be used at the planned flow rate
2. Set the upper pressure limit by adding the system pressure observed in step 1 to the column pressure rating.

If the system pressure is too high:

1. Check carefully for plugged or crimped tubing or other restrictions in your plumbing.
2. Use larger-ID or shorter tubing.
3. Use a larger detector flow cell.

In some systems, excessive system pressure can prevent the high flow rates required to take full advantage of Perfusion Chromatography technology.

It is important to isolate the relative contribution of column and instrument when pressures approach the maximum column pressure. Take the column out of line and determine the contributions of the column and the instrument using the following equation:

Measured Pressure = Column Pressure Drop + System Pressure

9.2 Check the Gradient System

High flow rates and short run times can expose both operational and design problems in gradient blending systems. Gradient system problems can affect step changes as well as linear gradients. Most problems come from one of two sources:

- Excessive delay volume (dwell) or mixing volume can cause both delay in the start of the gradient at the column and rounding or distortion of the edges of the gradient. Mixing or delay volume can be reduced by using a smaller mixer and shortening the tubing between the mixer and sample injector.
- Poor gradient proportioning can cause either short-term fluctuations or long-term inaccuracies. Adding a mixer can sometimes help.

The gradient can be visualized as follows:

1. Connect a union in place of the column.
2. Form a gradient with water as the A buffer and 0.5% acetone/water as the B buffer with detection set at 280 nm.

   ⚠ WARNING CHEMICAL HAZARD. Acetone *is a flammable liquid and vapor. It may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. It may cause central nervous system depression. Keep away from heat, sparks, and flame. Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.*

   The UV absorbance is directly proportional to the concentration of B buffer and can be compared to the programmed gradient.

Consult your system vendor if you encounter serious gradient problems.

9.3 Adjust the Data Collection System

Because Perfusion Chromatography runs are much shorter than conventional chromatography runs, you may need to adjust your data collection system as follows:

- To obtain high-definition chromatograms for analytical runs, use a shorter total run time and higher data collection rate (or lower average peak width parameter). A typical data collection rate is 10 points/second.
- If you use a chart recorder, increase the chart speed in proportion to the flow rate increase.

9.4 Maintain Your Column and System

Perfusion Chromatography allows you to perform runs more quickly than other chromatography techniques. Consequently, perform maintenance tasks such as replacing filters or regenerating columns after a certain number of runs rather than after a set period of time. You can reduce the frequency of such maintenance by always filtering the sample and mobile phases.

9.5 Think in Terms of Column Volumes

In any chromatographic separation, as flow rate increases, gradient time must decrease to maintain constant gradient volume. At the flow rates used for Perfusion Chromatography, the gradient times are dramatically shorter than what is typical for conventional chromatography. To convert a method to Perfusion Chromatography, keep the gradient volume constant in terms of column volumes and adjust the time of the gradient according to the new flow rate.

When you work routinely with Perfusion Chromatography, always think of gradients in terms of column volumes, because a slight change in gradient time may result in a dramatic difference in gradient volume and column performance.

For example, on a conventional 4.6 × 250 mm analytical column (volume 4.25 mL), a 45-minute run at 1 mL/min represents a 10.6 column volume gradient.

On a POROS 4.6 mmD/100 mmL column (volume 1.7 mL), a 5 mL/min flow rate translates into 3 column volumes/min (5/1.7). Therefore, a 10.6 column-volume gradient would be completed in 3.5 minutes.

9.6 Adjust the Sample Load

If the volume of your POROS column is different from the column you are currently using, adjust the sample volume or mass proportionally to keep the same load per unit volume of column.

9.7 Measure Recovery Properly

Quantitation (recovery) measurements using peak integration are comparable run-to-run only if the run conditions are kept nearly constant.

Flow rate affects the value of the integrated peak area for a given mass recovered, because the amount of material recovered in a peak is equal to the concentration (absorbance) times the volume. However, an integrator (or integration software) quantitates peaks by summing absorbance measurements over *time*. A change in flow rate is a change in the amount of volume over time.

Therefore, time integration does not allow comparison of recovery at different flow rates. Direct comparison of your POROS column results with previous results on conventional chromatography may lead to the incorrect conclusion that recovery is less on columns packed with POROS media.

There are several ways to compensate for limitations in time-based integration:

- Multiply the peak area by the flow rate. Unfortunately, because integrators often vary the data rate with expected peak width, this approach can give invalid results.
- Using peak height can give a general comparison, but bandspreading differences due to different efficiencies or gradient slopes can cause errors.
- Recovery may be compared accurately using peak integration at the same flow rate, but *doing this may not show important effects such as a recovery increase due to shorter time on the column with Perfusion Chromatography.*
- Collect the peaks carefully and analyze using spectrophotometry or other methods. Make sure to develop blanks, especially when UV-absorbing components are present in the eluent.
- If peak collection is not feasible, generate standard curves by injecting increasing amounts of calibration standards of known concentration. If the measured peak area increases linearly with load, and the standard curve passes through the origin, it is very likely that recovery is high.

10 Technical Support

Applied Biosystems is dedicated to helping you use Perfusion Chromatography technology and POROS media to the fullest extent possible. Our biochromatographers, bioprocess engineers, and applications development laboratories are available for support, ranging from telephone consultation to full-scale method development.

Applied Biosystems also offers a full line of other POROS media for Perfusion Chromatography in the reversed-phase, ion-exchange, affinity, and other chromatographic modes. Please contact your Applied Biosystems representative for technical and ordering information.

Applied Biosystems publishes a continuing series of Application and Technical Notes highlighting specific purification problems and technical aspects of Perfusion Chromatography. Please contact Applied Biosystems directly for a publications list.

For more details or for answers to questions on POROS 50 MC media, columns, Perfusion Chromatography, or other products, contact Applied Biosystems. Refer to the back page of this document for contact information.

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